Title:

Phase II clinical trial evaluating efficacy and safety of **Oral** immunotherapy with Third Generation Gc Protein derived Macrophage Activating Factor (Gc**MAF**) in hospitalized patients with **CO**VID-19 pneumonia: the **COral-MAF**1 Trial.

Study code: COral-MAF1

Promoter:	Ospedale del Mare, ASL Napoli 1 Centro Naples, Italy – Dr Lucrezia Spadera, MD, Department of Department of Otolaryngology-Head and Neck Surgery, Ospedale del Mare - ASL Napoli 1 Centro, Director: Prof. Giuseppe Tortoriello
Coordinator Center:	COVID CENTER**,** Department of General Medicine Ospedale del Mare, ASL Napoli 1 Centro Naples, Italy Director: Dr. Ernesto Grasso

CONTACTS

Principal investigator and study Author	
Dr Lucrezia Spadera	Dr Lucrezia SPADERA, MD Department of Otolaryngology-Head and Neck Surgery, Ospedale del Mare - ASL Napoli 1 Centro Director: Prof. Giuseppe Tortoriello Viale delle Metamorfosi, Ponticelli 80131 Naples, Italy Ph +39 3497518971 +39 08118775097 E-mail: *lucrezia.spadera@libero.it*

Co-investigators

Dr. Maria SPADERA, MD
Department of Anesthesiology and Intensive Care, San Giovanni Bosco Hospital - ASL Napoli 1 Centro
Ph +39 3297849758; e-mail: *maria.spadera@libero.it*

Dr Gabriella OLIVA, PhD
Department of General Medicine, Ospedale del Mare - ASL Napoli 1 Centro
Ph +39 08118775532 (office); e-mail: *gabriella.oliva@aslnapoli1centro.it*

Dr Marina LUGARÀ, MD
Department of General Medicine, Ospedale del Mare - ASL Napoli 1 Centro
Ph +39 08118775532 (office); e-mail: *marinalugara82@gmail.com*

Dr. Stefania TAMBURRINI, PhD
Department of Radiology, Ospedale del Mare - ASL Napoli 1 Centro
Ph +39 08118775205 (office); e-mail: *tamburrinistefania@gmail.com*

Dr Pietro Paolo SATURNINO, MD
Department of Radiology, Ospedale del Mare - ASL Napoli 1 Centro
Ph +39 08118775205 (office); e-mail: *pietropsaturnino@libero.it*

Dr Mariano CONTICELLI, Biologist
Department of Clinical Pathology, Ospedale del Mare - ASL Napoli 1 Centro
Ph +39 08118775094 (office); e-mail: *mariano.conticelli@aslnapoli1centro.it*

Dr. Maurizio D'AMORA, MD
Department of Laboratory Medicine and Clinical Pathology, Ospedale San Paolo – ASL Napoli 1 Centro, Via Terracina, 219 - 80125, Naples, Italy
Ph +39 0812547859; e-mail: *maurizio.damora@aslnapoli1centro.it*

Dr. Vincenzo BASSI, MD, Director
Department of General Medicine, "San Giovanni Bosco" COVID Hospital - ASL Napoli 1 Centro
Ph +39 3394227298; e-mail: vincenzo.bassi@aslnapoli1centro.it

Dr. Francesco CALDERARO, MD,
Department of General Medicine, "San Giovanni Bosco" COVID Hospital - ASL Napoli 1 Centro
Ph +39 3463346505; e-mail: framed64@gmail.com
Dr. Olimpia FATTORUSO, MD
Department of Clinical Pathology, "San Giovanni Bosco" COVID Hospital - ASL Napoli 1 Centro
Ph +39 0812545255 (office); e-mail: oli.fattoruso@gmail.com

Dr Mario CAPASSO, PhD, Associate Professor
University of Naples Federico II, Department of Molecular Medicine and medical Biotechnologies - Head of Bioinformatics Service for NGS - CEINGE Biotecnologie Avanzate, Via Gaetano Salvatore, 486 - 80145 Naples, Italy
Phone +39 0813737889 (office) / +39 0813737736 (lab.) / Fax +39 0813737804
E-mail: mario.capasso@unina.it

Dr Roberta RUSSO, PhD, Assistant Professor
University of Naples Federico II, Department of Molecular Medicine and medical Biotechnologies - CEINGE Biotecnologie Avanzate
E-mail: *roberta.russo@unina.it*

Dr Immacolata ANDOLFO, PhD, Researcher
University of Naples Federico II
Department of Molecular Medicine and medical Biotechnologies - CEINGE Biotecnologie Avanzate
E-mail: *immandolfo@gmail.com*

PLASMA/SERUM PROTEIN EVALUATION

Dr Roberta MARRA, Pharmacist
Department of Pharmacy, Ospedale del Mare - ASL Napoli 1 Centro
Ph +39 3452837797; e-mail: *roberta.marra@aslnapoli1centro.it*

PHARMACOVIGILANCE

SUMMARY

1. Protocol Synopsis

Full Title	Phase II clinical trial evaluating efficacy and safety of **Oral** immunotherapy with Third Generation Gc Protein derived Macrophage Activating Factor (Gc**MAF**) in hospitalized patients with **CO**VID-19 pneumonia: the **COral-MAF**1 Trial
Short title	Saisei Colostrum-MAF in the treatment of hospitalized patients with COVID-19 pneumonia
Protocol Code	**COral-MAF1**
Protocol number	
Promoter	Ospedale del Mare, ASL Napoli 1 Centro - Naples, Italy Dr Lucrezia Spadera, MD, Department of Otolaryngology-Head and Neck Surgery, Ospedale del Mare - ASL Napoli 1 Centro Director: Prof. Giuseppe Tortoriello
Coordinator Center	COVID Center, Ospedale del Mare, ASL Napoli 1 Centro - Naples, Italy
Other Investigational Centers	Department of General Medicine, "San Giovanni Bosco" COVID Hospital - ASL Napoli 1 Centro
Phase	II
Investigation type	Drug
Study type	Interventional
Purpose and rationale	To evaluate the efficacy and safety of immunotherapy with oral colostrum-MAF plus standard-of-care therapy in hospitalized patients with COVID-19- induced pneumonia. As of August 16, 2020, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been responsible for more than 21 294 000 infections and about 760 000 deaths worldwide. Mortality rate has been reported to be approximately 3.7%, which is nearly 4 times higher than that of influenza: there is an urgent need for effective treatment. Accumulating evidence suggests that patients with severe acute COVID-19 pneumonia have a cytokine storm syndrome, or unbalanced hyper-inflammatory response. The main substance of SaiSei MAF products is group-specific component macrophage activating factor (GcMAF), a protein which results from sequential deglycosylation of its precursor - vitamin D-binding protein (VDBP). The group-specific component (Gc) protein - VDBP produced in the liver. It has been reported to have multifunctional properties as a transporter of serum vitamin D3 and its metabolites, function as an actin scavenger during cellular injury, bind fatty acids, and act as a

	chemotaxin for phagocytic cells, and also play a role in macrophage activation as a precursor for GcMAF. It is now well known that GcMAF plays a crucial role in immune system regulation as a primary defense against infections. Thus, this multifunctional protein, released into the blood stream, acts as a systemic immune modulator without pro-inflammatory activities. In an animal study, IL-6 level was shown to be dramatically decreased after 21 days of oral administration colostrum MAF. Indeed, data from previous studies and clinical practice have been reported its effectiveness and safety in the treatment of many pathologies such as HIV infection and other infectious diseases, some types of cancer, juvenile osteopetrosis, immunological, and neurological diseases. These observations suggest that oral immunotherapy with colostrum-MAF is potentially an effective and well-tolerated treatment for COVID-19 pneumonia. In addition, gastrointestinal involvement is well known in coronavirus infections of animals and humans. The angiotensin-converting enzyme II (ACE2), the entry receptor for SARS-CoV, is highly expressed in proximal and distal enterocytes that are directly exposed to foreign pathogens. It considers the mechanism of SARS-CoV-2 can actively infect and replicate in the gastrointestinal tract. SARS-CoV-2 indirectly damages the digestive system through a chain of inflammatory responses. Delivered topically to the small intestine by an acid-resistant enteric-coated capsule colostrum MAF can directly activate a large number of gut mucosal macrophages for virus control, localizing intestinal inflammation and resolving through driven phagocytic scavenger function. Macrophages in the gastrointestinal mucosa represent the largest pool of tissue macrophages in the body, which besides the local functions are directing the systemic immune response.
Primary objective	To evaluate the efficacy of oral MAF for the treatment of COVID-19 pneumonia on the basis of the rate of transfer to the intensive care unit (ICU) or mortality rate.
Secondary objectives	- Imaging progression on chest CT - Lung Ultrasound Imaging (LUS) progression - duration of hospital stay, expressed in days; - days on non-invasive ventilation; - days on mechanical ventilation;

	- days in ICU; - days with use of supplemental O_2; - discharge rate at day 28; - clinical evolution; - time to resolution of fever; - progression of respiratory failure; - changes from baseline in: white blood cell count (WBC), hemoglobin, platelets, CRP, ESR, LDH, procalcitonin, IL-1, IL-6, TNF-α, D-dimer, and fibrinogen; - changes from baseline in: MAF precursor activity of serum Gc protein and serum nagalase activity, as markers for response to treatment; - kinetic changes of viral loads detected in nasopharyngeal swabs; - number of Serious Adverse Events (SAE) and Adverse Drug Reaction (ADR) (expected and unexpected); - patient compliance with treatment.
Protocol design	Phase II, interventional, prospective, multicenter, non-profit study**.**
Study population	The study population includes adult male and female patients who are hospitalized and diagnosed with COVID-19-induced pneumonia.
Inclusion criteria	• Adults (≥ 18 years of age); • signed informed consent by any patient capable of giving consent, or, when the patient is not capable of giving consent, by his or her legal/authorized representative or according to local guidelines; • patients clinically diagnosed with SARS-CoV-2 virus by PCR or by other approved diagnostic methodology; • hospitalized with COVID-19-induced pneumonia evidenced by chest x-ray or CT scan with pulmonary infiltrates; • patients having a PAO2/FIO2 ratio > 250 mmHg; • well-selected patients having a PAO2/FIO2 ratio ≤ 250 mmHg that, in the investigator's judgment, doesn't preclude the patient's safe participation in and completion of the study; • patients being able to swallow.
Exclusion criteria	• Proportion of hospitalized patients requiring invasive mechanical ventilation at the time of

	hospital admission (patients requiring non-invasive mechanical ventilation are eligible); • uncontrolled systemic infection (other than COVID-19); • hypersensitivity to the active substance or to any of the excipients of the experimental drug, including known allergy to dairy product; • any serious medical condition or abnormality of clinical laboratory tests; • in the opinion of the investigator, progression to death is imminent and highly likely within the next 24 hours, irrespective of the provision of treatments; • current participation in any other interventional investigational trials; • pregnant or breastfeeding woman; • concurrent malignancy requiring chemotherapy; • renal insufficiency; • all types of disability.
Safety assessments	Adverse event monitoring, physical examinations, and monitoring of laboratory safety values.
Sample Size Estimation and Statistical design	Up to approximately 97 subjects will be enrolled. The analysis of all primary and secondary endpoints will be based on the Evaluable Population which includes all enrolled subjects who will receive at least one dose of study drug. Descriptive statistics will be used to describe the basic features of the data in the study. Kaplan–Meier survival curves will also be provided to analyze "time-to-event" data. Longitudinal data analysis with mixed models will be used to evaluate laboratory findings. Incidence rate of treatment emergent adverse events will be reported per patient. Descriptive statistics will be done for safety assessments.
Study duration	The follow-up according to the protocol is 28 days for each patient enrolled. The end of the study is expected at 1 month from the last follow-up of the last patient enrolled. The study will be performed in approximately 3 months starting from the first patient enrolled (depending on the speed of enrollment).
Keywords	COVID-19, acute respiratory failure, colostrum-MAF, SARS-CoV-2

2. Background and rationale

2.1 Background

In December 2019, a new pandemic occurred that the novel coronavirus SARS-CoV-2 was causative agent, emerged from Wuhan, China.[1] Within few weeks, the disease had spread worldwide and the World Health Organization (WHO), on March 11, 2020, declared COVID-19 a pandemic with a significant impact on health care provision and associated costs.[2]

Real-time reverse transcriptase–polymerase chain reaction (rRT-PCR) testing of nose and throat swab has been recommended as the confirmatory test for COVID-19.[3] Other alternative samples for rRT-PCR include bronchoalveolar lavage or endotracheal aspirate.

Based on clinical reports, it is noteworthy that COVID-19 causes various degree of illness ranging from asymptomatic or milder symptomatic cases to severe lung injury or even multiorgan dysfunction with liver and kidney impairment.[4-7] Even in not severe patients, the heterogeneity of symptoms is consistent with the increasing evidence that SARS-CoV-2 shows a broad tissue tropism, being able to attack almost anything in the body.

The largest report of COVID-19 from the Chinese Centers for Disease Control and Prevention summarized findings from 72,314 cases and reported that although 81% of them showed a mild clinical picture with an overall case fatality rate of 2.3% and a small sub-group of 5% presented with respiratory failure, septic shock, and multiorgan dysfunction, requiring intensive care management and resulting in fatality in 50% of such cases.[8] The initial stage of infection, characterized by mild and flu-like symptoms, occurs at the time of inoculation and viral multiplication in the respiratory system (stage I, mild).[9] Stage II disease (moderate) is mainly associated with localized inflammation in the lung. A minority of cases may enter into the third stage of COVID-19 (Stage III, severe), which resembles an extrapulmonary systemic hyperinflammation syndrome. During this phase, a significant release of proinflammatory cytokines and biomarkers into the blood occurs, just like in the "cytokine storm syndrome".[9]

Critically ill patients with SARS-CoV-2 are most likely to benefit from intensive care treatment. Indeed, recent epidemiological studies on COVID-19 hospitalized patients have been showed a rate of transfer to the Intensive Care Unit (ICU) equal to 20-26%.[10,11] Moreover, the same Authors have been reported that 50% of all patients admitted to the ICU has died.[12]

Because of the primary involvement of the respiratory system, chest CT is strongly recommended in suspected COVID-19 cases, for both initial evaluation and follow-up.[13] It has well been documented that CT findings may be present even before symptom onset.[14,15] The most common

patterns seen on chest CT were ground-glass opacity, interlobular septal thickening, air bronchogram, bilateral patchy shadowing, crazy-paving pattern, and thickening of the adjacent pleura, resembling an interstitial involvement in viral pneumonia.

CT findings have proven to be diagnostic in a number of cases with an initial false-negative rT-PCR screening test.[16,17] So, high-resolution CT (HRCT) is of outstanding importance as it is the main tool for screening, primary diagnosis, and evaluation of disease severity. In particular, CT visual quantitative evaluation based on CT images has high consistency and high diagnostic ability, which can reflect clinical classification and guide the clinical treatment by combining with the clinical information.[18]

Pan Y et al.[11] have found that more than 85% of patients showed imaging signs associated with disease progression within 3–14 days after an initial CT study. Indeed, it has been well assessed by chest CT findings that COVID-19 may get worse over a short period of time (about 7 days after clinical manifestations started) with an early evidence of increased ground glass density patches and a fused and large-scale light consolidation and air-bronchograms.[13]

Under the light of microscope, the lungs revealed diffuse alveolar damage with formation of numerous hyaline membranes, very patchy and sparse interstitial chronic inflammation composed mainly of lymphocytes, thrombi within a few small pulmonary artery branches, congestion of alveolar septal capillaries, focal edema fluid, and macrophage infiltration within the airspaces.[19-22]

The more significant laboratory abnormalities were metabolic acidosis, lymphocytopenia, leukopenia, thrombocytopenia, elevated levels of C-reactive protein (CRP), interleukin-6 (IL-6), lactate dehydrogenase (LDH) and D-dimer.[23,24]

Over the last five months, there have been increasing numbers of reports that struggle to understand the pathogenesis of the coronavirus disease 2019 (COVID-19) pandemic. To date, the most commonly investigated hypothesis about the underlying mechanisms of multi-organ failure may be summarized into three main targets: microcirculation dysfunction, overwhelming inflammation and abnormal coagulation.[24]

Clinical, radiologic and laboratory findings, as well as preliminary autopsy studies, seem to support this hypothesis. As firstly suggested by Huang C et al.,[6] the systemic cytokine storm could play a key role in the virus-induced tissue damage.

However, the question “what the link for the overproduction of pro-inflammatory mediators and the immune suppression, on the hand, and microvascular injury and thromboembolism, on the other, is”, remains unclear.

Being the knowledge of this issue very scarce, lessons learned from other human pathogenic viruses, with specific reference to human immunodeficiency virus (HIV), could be diriment.
Unfortunately, no drug or vaccine has yet been approved to treat human coronaviruses and new interventions based on drugs directly active on the virus itself are likely to require months to years to develop. The main targets of the pharmacologic approaches to COVID-19, especially for the complicated cases, are addressed to modulate the immune system and counteract the overwhelming inflammation. Notably, the mechanisms we have hypothesized about the possible pathogenesis of the cell and tissue damage induced by SARS-CoV-2 seem to provide a common denominator in explaining the effects of most drugs currently in use in the clinical trials: these include antivirals, immunomodulating and/or anti-inflammatory drugs.[25,26]
In particular, based on their antiviral activity,[27] chloroquine and hydroxychloroquine, initially conceived as antimalarial therapeutics, were proposed to treat hospitalized patients with COVID-19, with or without azithromycin, showing promising efficacy in "inhibiting the exacerbation of pneumonia, improving lung imaging findings, promoting a virus negative conversion and shortening the disease course".[28,29] On the other hand, hydroxychloroquine is the cornerstone of medical therapy in lupus, where it acts as an immunomodulatory without immunosuppressive effects.[30] However, because of the lack of evidence about the efficacy and safety of these drugs[31], the Italian Medicines Agency[32] on July 17 said it had withdrawn an emergency approval for use of the malaria drug hydroxychloroquine or antivirals as a Covid-19 treatment out of clinical trials. Meanwhile the use of low-molecular-weight heparin for COVID-19 is restricted only to well selected hospitalized patients.
Tocilizumab, an IL-6 antagonist, approved for the treatment of rheumatoid arthritis and juvenile idiopathic arthritis, also had initial therapeutic application in critical COVID-19 patients, providing encouraging results.[33] However, the phase III clinical trial (COVACTA)[34] for evaluating tocilizumab in hospitalized patients with severe COVID-19 pneumonia found no difference between tocilizumab versus placebo in intensive care requirements or mortality.
The rationale basis for the use of monoclonal antibodies in patients affected by SARS-CoV-2 seems to lie in the so-called systemic cytokine storm. Taking into account the key role of VEGF in enhancing angiogenesis in acute lung injury and ARDS,[35] two trials, evaluating the efficacy of bevacizumab as VEGF antagonist in the treatment of COVID-19 (BEST-PC and BEST-RCT), were also started.[36,37]

In light of pathological findings of pulmonary inflammation with edema and hyaline membrane formation, timely and appropriate use of drugs with understood safety profiles aimed at reducing inflammation, microcirculatory dysfunction, oxidative stress, neoangiogenesis and

microthrombotic occlusion, in a targeted way, together with ventilator support, should be considered for the severe patients to prevent and treat ARDS development.

2.2 Rationale for Oral immunotherapy with Saisei Colostrum-MAF

It is now well known that GcMAF plays a crucial role in immune system regulation as a primary defense against infections.[38-44]

Based on the aforementioned findings and on documented analogies between SARS-CoV-2 and HIV,[45] we hypothesized that the reduced conversion activity of the Gc protein (human group-specific component (Gc)) into the macrophage activating factor (MAF) could have a key role in the dysregulate immune response induced by SARS-CoV-2, just like for HIV infected patients.[38,39] If this hypothesis is correct, it might help to set a valid strategy of immunotherapy also based on an off-label use of GcMAF in critically ill COVID-19 patients.

2.2.1 Gc globulin, DBP and GcMAF: three in one

Serum Gc protein, also known as vitamin D-binding protein (DBP), is a multifunctional protein present in plasma/serum at concentrations of 300-600 mg/L.[40] It carries a trisaccharide consisting of N-acetylgalactosamine with dibranched galactose and sialic acid termini at 420 threonine residue.[41] Stepwise hydrolysis of Gc protein by the inducible membranous β-galactosidase of stimulated B-lymphocytes, and by the Neu-1 sialidase of T-lymphocytes converts it into the active GcMAF.[41-43] On the contrary, deglycosilation of Gc protein by action of the enzyme alpha-*N*-acetylgalactosaminidase, named nagalase, secreted from HIV-infected cells leads to lack of macrophage activation and to immunosuppression, as a consequence.[38,39] It is remarkable that nagalase was demonstrated to be an intrinsic component not only of the envelope glycoproteins gp120 and gp160 of HIV but also of the hemagglutinin (HE) of influenza virus[39,44] and even produced by neoplastic cells.[46-48] Indeed, flu-like symptoms with serum nagalase activity similar to the influenza acute state were reported in the early stage of HIV-infection, so that the serum enzyme activity may be detectable at all phases of HIV-infection,[38,39] Similarly, most COVID-19 patients complained of flu-like symptoms in the early stages of the disease.[4-7]

2.2.2 The role of GcMAF as a multifunctional immune modulator and possible implications in Covid-19

In addition to the storage and transport of active vitamin D3, GcMAF's effects include macrophage modulation, osteoclast activation, facilitation of neutrophil chemotaxis mediated by C5 derived peptide, superoxide activity, scavenging of circulating G-actin, anti-angiogenetic and anti-tumor

properties.[49-53] Thus, this multifunctional protein, released into the blood stream, acts as a systemic immune modulator without pro-inflammatory activities. This means that any function impairment of Gc-globulin could result in a state of both immunosuppression and uncontrolled inflammation, just like in severe COVID-19. Interestingly, HIV viremia was associated with higher level of biomarkers of inflammation (measured by IL-6), monocyte activation (soluble CD14), and coagulation (D-dimer), leading to increased mortality, as compared with uninfected people.[54] Meanwhile, in COVID-19 patients, in addition to the reduced peripheral lymphocyte counts, mainly $CD4^+$ T and $CD8^+$ T cells, there were found significant high levels of pro-inflammatory cytokines and chemokines.[4-8,23] Indeed, GcMAF is not only a simple potent activator for macrophages, but more specifically is able to turn macrophage activity on at the sites of infection/inflammation and then to induce their apoptosis by upregulating caspase activity via the p38 and JNK1/2 pathways when no longer needed.[55] Post-mortem lung observations of patients died of COVID-19 showed the presence of mononuclear cells and macrophages infiltrating air spaces by autopsy.[19-22]

With regards to the anti-oxidant properties, it was assessed that GcMAF promotes the superoxide generating capacity of activated macrophages and the production of nitric oxide (NO).[56] An article by Nozik-Grayck et al.[57] pertinently and interestingly showed that the expression of extracellular superoxide dismutase (EC-SOD) mRNA and protein is cell- and tissue-specific and is prominent in lung, heart, blood vessels, placenta and kidney. In particular, high levels of EC-SOD are present in lung macrophages, alveolar type II cells, fibroblasts, vascular smooth muscle cells, and endothelial cells. EC-SOD limits oxidative stress and preserves NO bioactivity, thus protecting against a number of lung and cardiovascular diseases.[57] Even though only in a minority of cases, COVID-19 may progress to life-threatening complications, including respiratory failure, acute cardiac injury, acute kidney injury, septic shock, disseminated intra-vascular coagulation (DIC), and multi-organ dysfunction.[4-7] Hypoxemia was found to be associated with interstitial pneumonia and, in 10% to 20% of cases, developed into acute respiratory distress syndrome (ARDS).[4-7]

In this connection, it was documented that ARDS as well as organ dysfunction and septic shock is characterized by actin release which is involved in microvascular impairment.[58,59]

DBP has an additional function in binding monomeric globular (G)-actin with high affinity. Thereby, rapidly removing polymeric actin fibrils from the blood stream, it prevents actin polymers from clogging the micro vessels not unlike fibrinogen/fibrin and consequently platelet aggregation and micro thrombi formation.[60,61] What we postulated could also explain hypercoagulability with elevated concentrations of D-dimer, fibrin degradation products increase, PT and aPTT prolongation, observed in COVID-19 patients.[62,63] Tang N et al.[63] reported that 71.4% of the non survivors of COVID-19 matched the grade of overt-DIC according to the

International Society on Thrombosis and Haemostasis (ISTH) diagnostic criteria for DIC. Murine models deficient in DBP showed lung damage caused by actin polymerization, developing severe acute lung inflammation with vascular leakage, hemorrhage and thickening of the vascular wall after actin injection.[64] Interestingly, the lung was the only organ that showed inflammatory injury after intravenous actin injection. The observed lung inflammation was consistent with alterations to lung microvascular endothelial cells. Indeed, when lung endothelial cells were exposed to DBP-actin complexes in vitro showed enhanced cell death.[64] Reduced levels of DBP were even observed in sepsis and organ dysfunction of trauma patients as well as complete depletion of free DBP in those affected by septic shock.[58,59] These data could provide support for pathogenic explanations of cellular and tissue damage by SARS-CoV-2 and, at the same time, for the therapeutic use of DBP to bind extracellular actin and counteract microcirculatory alterations.

Whereas DBP also binds free fatty acids, it was shown that the administration of GcMAF complexed with oleic acid (OA) via nebulisation or subcutaneous injection led to rapid decrease of blood pressure and increase in splenic blood flow, as a result of a verisimilar synergistic NO release by OA-GcMAF-activated alveolar and splenic macrophages.[56] Severe or critically ill COVID-19 patients developed clinical typical manifestations of shock, even in the absence of overt hypotension.[24]

Furthermore, it was found that GcMAF can inhibit the angiogenesis induced by pro-inflammatory prostaglandin E1,[65] which serves roles in the promotion of vascular endothelial growth factor (VEGF) expression.[66] A key role of VEGF in acute lung injury and ARDS was confirmed.[67]

2.2.3 Explaining the clinical heterogeneity of Covid-19 with DBP polymorphisms, estrogens and vitamin D

Reflecting the fact that clinical features and severity of symptoms vary widely between and within each COVID-19 patient, with older males more likely to be affected and in a more severe manner,[68] we sought to relate it with some special feature of DBP. Several studies showed that the polymorphisms of DBP were associated with susceptibility or resistance to disease states including chronic obstructive pulmonary disease.[69,70] Moreover, whereas androgens were not found to have any effect on circulating levels of DBP, exposure to high levels of estrogens increased them by up to 50%, suggesting a potential protective role of estrogens against COVID-19.[53] On the other hand, in relation to vitamin D status, advanced age was recognized as one of the major risk factors for vitamin D deficiency.[71] Animal-based studies also demonstrated that deficiencies in both dietary protein- and energy-intake decreased the concentration of DBP in the circulation.[72] These data seem to be in line with the growing evidence that vitamin D supplementation could reduce the risk of COVID-19 infections and deaths.[73-75]

2.3 Previous clinical experience of immunotherapy with GcMAF

Although the administration of GcMAF is a yet an unapproved therapy, data from previous studies and clinical practice reported its effectiveness in the treatment of many pathologies such as HIV infection[76] and other infectious diseases,[77] some types of cancer,[78-82] juvenile osteopetrosis,[83] immunological (systemic erythematous lupus),[84] and neurological (multiple sclerosis, autism) diseases.[79,85,86] In the same conditions, it was found an inverse correlation between the MAF precursor activity and serum levels of nagalase (reference ranges from 0,32 to 0,65-0,95 nM/min/ng), therefore showing to be other than pathogenicity or cancer biomarkers, also good prognosticators of illness and response to therapy.[76-86]

With specific reference to immunotherapy of HIV-infected patients with GcMAF, Yamamoto et al.,[76] the pioneers in this field, have published data showing the success of this approach with eradication of HIV-infection after less than 18 weekly administrations of 100 ng of intramuscular GcMAF. However, it should be noted that this paper was withdrawn by the same authors because "irregularities in the documentation for institutional review board approval" occurred independently from the validity of their clinical observations.

2.4 GcMAF – The state of art

2.4.1 Description of the active substances

GcMAF - the macrophage-activating lymphokine

The main substance of SaiSei MAF products is group-specific component macrophage activating factor (GcMAF), a protein which results from sequential deglycosylation of its precursor - vitamin D-binding protein (VDBP). The group-specific component (Gc) protein - VDBP produced in the liver. It has been reported to have multifunctional properties as a transporter of serum vitamin D3 and its metabolites, function as an actin scavenger during cellular injury, bind fatty acids, and act as a chemotaxin for phagocytic cells, and also play a role in macrophage activation as a precursor for GcMAF. The structure of Gc protein is highly homologous to serum albumin and it has a triple-domain modular structure termed as domains I, II and III. Domain III of Gc protein (C-terminal end) harbors a single glycosylation site.[49] The terminal *N*-acetylgalactosamine (GalNAc) moiety in domain III is the region involved in the GcMAF mediated macrophage activation cascade. During inflammation, lysophosphatidylcholine is released from tissue which induces the expression of beta-galactosidase in B cells and sialidase in T cells. These enzymes hydrolyze terminal galactose and sialic acid saccharides of Gc protein to convert it into GcMAF with an *N*-acetylgalactosamine moiety.[49] GcMAF can be produced *ex vivo* by exposing isolated Gc protein

or the biological fluids bovine milk, whey and human serum to beta-galactosidase and sialidase treatment.

Bovine colostrum

Bovine colostrum powder contains serum proteins, including Gc protein and other glycoproteins, albumin, insulin-like growth factor, epidermal growth factor, nerve growth factor, vitamin D, lactoferrin, and main immunoglobulins G, A and M.

2.4.2 Evolution of production technology

Purified GcMAF

Gc protein has a high affinity for the 25-hydroxyvitamin D3 [25(OH)D3] J, Ka = 5 x 10^8 M^{-1} and can be purified from human serum using a 25(OH)D3 affinity column chromatography. The artificial treatment with beta-galactosidase and sialidase applied to the purified Gc protein have been commonly used for producing the first generation GcMAF - the serum-purified GcMAF.[87] The technology had significant problems. The final product contamination was difficult to avoid when a chromatography column was used repeatedly. When at room temperature - in an environment with oxygen, and in the absence of antioxidants, such as albumin and uric acid, which are present in blood, the serum-purified GcMAF protein has been shown to have a low stability.

Serum MAF - VitD-GcMAF containing human serum/plasma

In second-generation GcMAF- Serum MAF, whole human serum/plasma acts as a substrate for the enzymatic treatment.[88] Saisei Pharma have developed technology where Gc protein purification step via the 25(OH)D3 affinity column chromatography is avoided due to the following reasons:

1. The chromatography column is not a disposable device and thus it is not a feasible method for clinical use due to the existing contamination and cross-contamination risk.
2. Using a chromatography column assumes also the use of certain detergents or the exposure to acidic or alkaline conditions to extract the Gc protein bound to the column. As a result, impurities enter the final product.

It was decided to reproduce *ex-vivo*, the physiological process of conversion of the VDBP to the GcMAF by bringing autologous patient's serum or commercial donors plasma into contact with β-galactosidase and sialidase under experimentally determined conditions. Since 1991 there is only one Gc 1f1f out of six existing subtypes of the Gc protein family that have been used for GcMAF conversion by Yamamoto and others, despite the fact that other subtypes also can be GcMAF

precursors. To achieve maximum activity of the final product, all six genotypes of Gc protein are targeted to be converted to GcMAF when the whole plasma pool undergoes enzymatic treatment. The data demonstrates that the technology results in the high biological activity of GcMAF containing human serum/plasma;[89-91] with the sustained macrophage activation seen in only a few minutes after *in vitro* exposure,[89] in contrast, it required 3 to 12 hours exposure for purified GcMAF. 10 ng GcMAF containing human serum corresponds to 10 ng of purified GcMAF on its effect on phagocytic index of mouse peritoneal macrophages in an *in vitro* experiment.[91]

Stability**:** this technology allows to keep stability and activity of GcMAF containing human serum for 14 days at room and for two years at a temperature of minus 20°C; whereas the stability, and as a result, the functional activity of purified GcMAF is decreasing at room temperature by over 50% in the first 5 hours. To keep its functionality, purified GcMAF needs to be stored at minus 80°C.[92]

Colostrum MAF - Bovine colostrum containing VitD- GcMAF complex

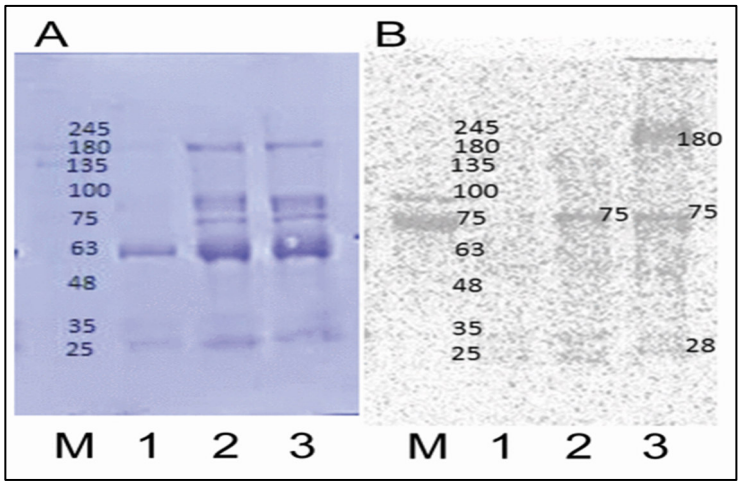
It was then hypothesized and proven that containing bovine serum proteins, including Gc and other glycoproteins bovine colostrum and whey could be substrates for β-galactosidase treatment to acquire macrophage-activation activity. Products were designated to enhance mucosal Immunity of intestine.

Identification of degalactosylated/desialylated bovine colostrum proteins

Preparation of degalactosylated/desialylated bovine colostrum and GcMAF-containing human serum. Bovine colostrum was obtained from Jun Sei Co. Ltd. (Tokyo, Japan). One milligram of bovine colostrum powder was dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 7.0) and incubated with 65 mU of β-D-galactosidase (from *Escherichia coli*; WAKO Pure Chemical Industries, Ltd., Osaka, Japan) either with or without 65 mU of neuraminidase (sialidase from *Clostridium perfringens*; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 h. The reaction mixture was then heated at 60°C for 10 min to deactivate the enzymes. The protein concentrations were determined using a Pierce® BCA protein assay kit. GcMAF-containing human serum was prepared as previously reported by Kuchiike et al.[91]

SDS-PAGE and western blotting. Degalactosylated/desialylated bovine colostrum was subjected to sodium dodecyl-polyacrylamide gel electrophoresis and, subsequently, electroblotted onto a nitrocellulose membrane. Non-specific binding was blocked by overnight incubation in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and 1% BSA at 4°C. The membranes were then probed with biotin-conjugated *Helix pomatia* agglutinin (HPA) lectin specific for GalNAc moiety. After membrane washing, the blots were incubated with horseradish peroxidase (HRP)-labeled

streptavidin as a secondary antibody. The blots were developed using an ECL western blotting detection system. The visualization and quantification of the Western blot bands were achieved using an ECL West. blotting detection system, a LumiCube chemiluminescence analyzer and JustTLC image analysis software.[93]



Picture 1.

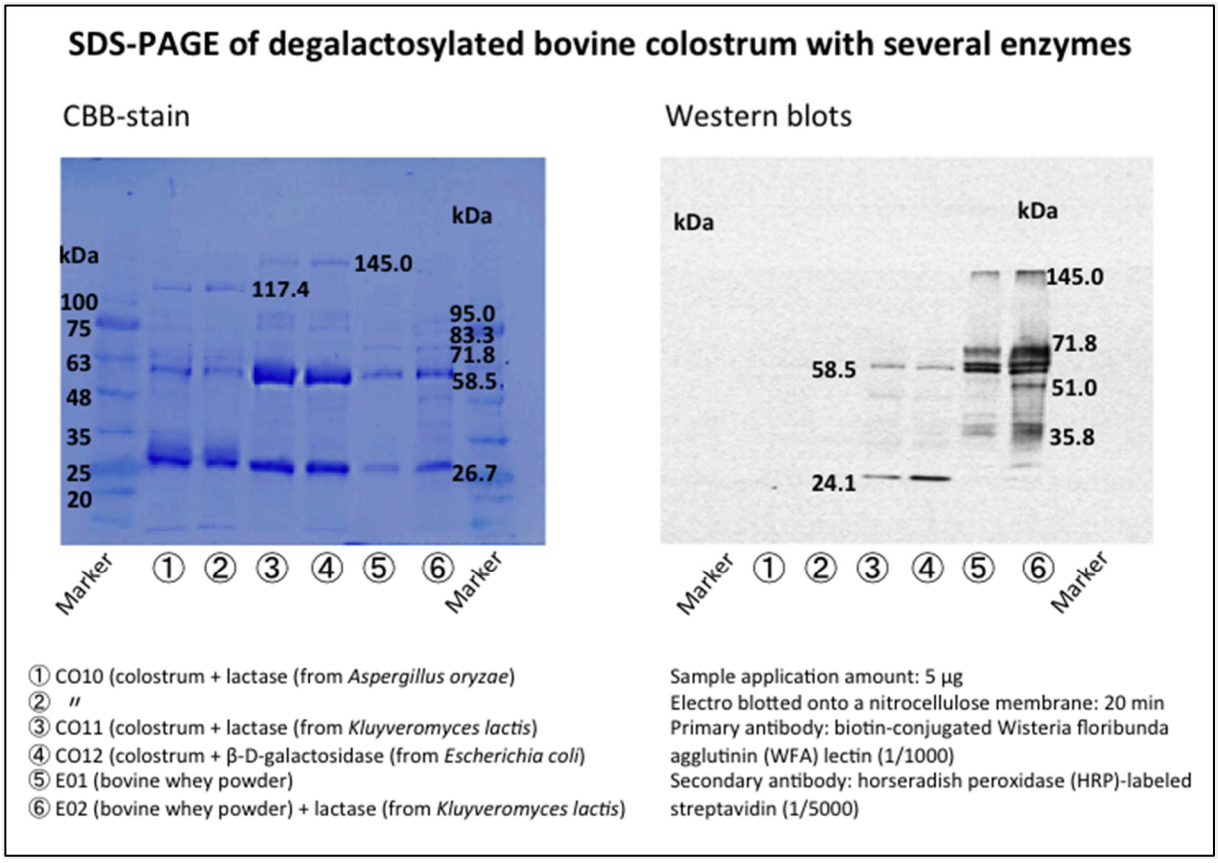
SDS-PAGE of degalactosylated and degalactosylated/desialylated bovine colostrum

(A) CBB-stain and (B) western blots probed with anti-human Gc globulin and Helix pomatia agglutinin (HPA) lectin. M, Marker; lane 1, bovine colostrum; lane 2, degalactosylated bovine colostrum; lane 3 degalactosylated/ desialylated bovine colostrum.

Preparation and identification of degalactosylated/desialylated bovine colostrum. We first checked the digestion activity of the *O*-linked sugar chain of the glycoprotein included in the bovine colostrum. Picture 1A shows the Coomassie Brilliant Blue (CBB) stain and Picture 1B shows the western blot of the bovine colostrum (lane 1), degalactosylated bovine colostrum (lane 2) and degalactosylated/desialylated bovine colostrum (lane 3). Five bands (180, 90, 75, 63, 28 kDa) were detected on the CBB stain, but only three bands (180, 75, 28 kDa) were detected by using an HPA lectin, which recognizes the GalNAc moiety. It suggests that the 75-kDa band corresponds to glycoprotein-α constituting IgA and the 28-kDa band corresponds to VDBP.[65,94]

Comparison study of Identification of degalactosylated bovine colostrum and milk proteins under different enzymes treatment.

Picture 2.



The lactase enzyme preparation from *Kluyveromyces marxianus* and the β-D-galactosidase from *Escherichia coli* were found to be most efficient (lane 3,4) base on corresponded to GcMAF protein band 58.5 kDa detected on both CBB stains and western blot where HPA lectin which recognizes the GalNAc moiety have been used.

2.4.3 In vitro studies of Immunomodulatory activity of degalactosylated/desialylated bovine colostrum (colostrum-MAF) and degalactosylated/desialylated human serum (serum-MAF)

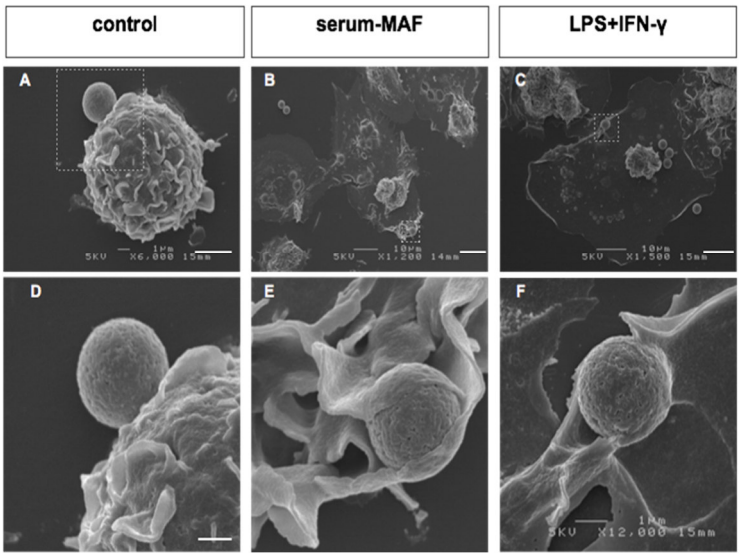


Figure 1

Morphological changes of THP-1-derived macrophages following MAF treatment *After a 4-h treatment with 8.17 µg/ml serum-MAF (B, E), 100 pg/ml LPS + 20 ng/ml IFN-γ (C, F), and control (A, D), beads were added to macrophages and fixed for SEM observation. Low magnification, whole cell images (A-C) and enlargements of the engulfing region (white rectangle) are shown (D-F). Scale bars; 10 µm in A-C, 1 µm in D-F.*[89]

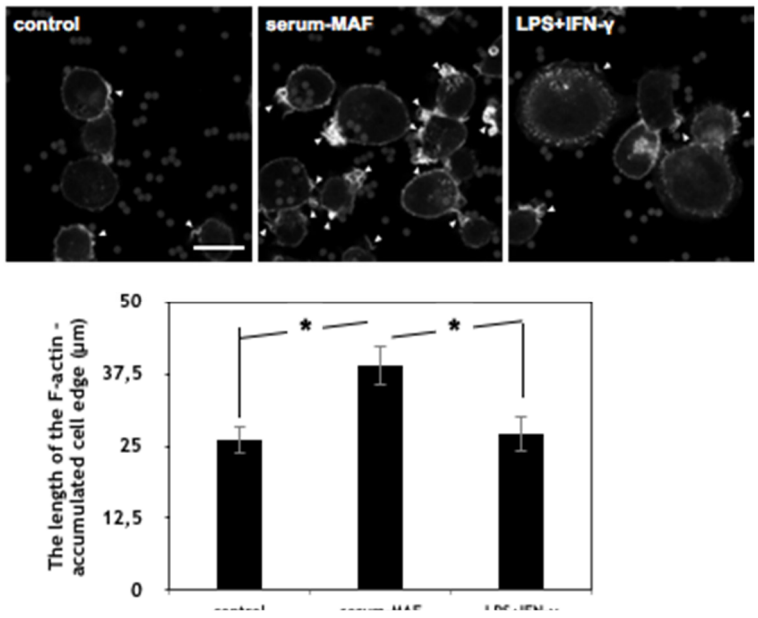
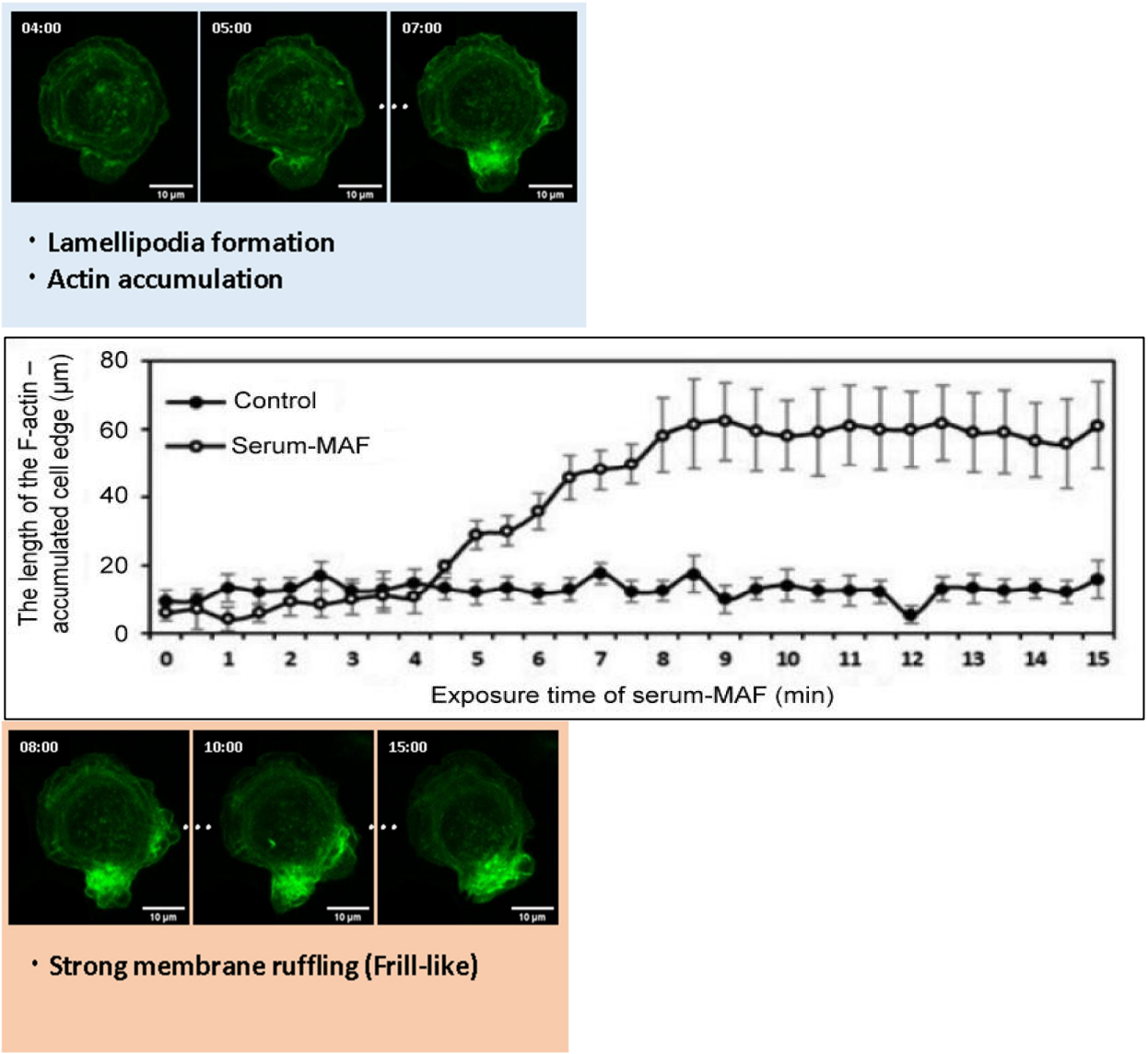


Figure 2

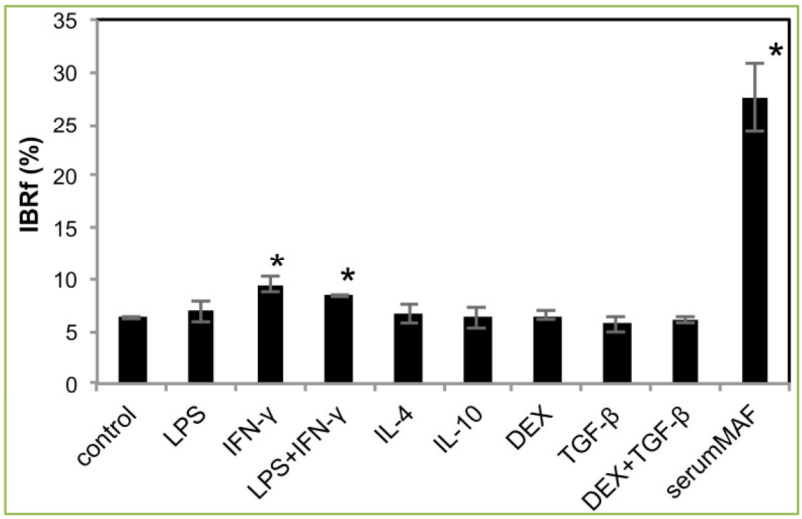
Differences in actin accumulation at the edge of the cells *Confocal images of Lifeact-THP-1 derived macrophages, treated with 8.17 µg/ml serum-MAF (B), 100 pg/ml LPS + 20 ng/ml IFN-γ (C), or without MAF (A) are shown. White arrowheads indicate actin accumulations in lamellipodia tips. Three-dimensional analysis of serum-MAF activated macrophage (D) represents intricate membrane ruffling at the site of actin accumulation. Scale bar; 10 µm. Actin accumulation was quantitatively analyzed using these images (E).*[89]

Figure 3



Time lapse images of Lifeact-THP-1 derived macrophages after the start of treatment with serum MAF compared to control are indicated in the graph. Actin accumulation was quantitatively analyzed. [89]

Figure 4



The comparison of different biological stimuli on phagocytic activity human cell line THP-1 derived macrophages

*The macrophage activation factors used in 1 μg/mL LPS, 20 ng/mL IFN-γ, 20 ng/mL DEX, 20 ng/mL TGF-β, 20 ng/mL IL-4, 20 ng/mL IL-10, 6 μg/mL serum MAF, and some mixtures of two of them. After MAFs activated macrophages, we evaluated their phagocytic activity. *p < 0.05 (two-tailed t test).*

Result serum MAF had a 2.9-fold higher macrophage activation rate than IFN-γ.[90]

Phagocytic activity mouse peritoneal macrophages under in vitro exposure to the pure, degalactosylated and degalactosylated/desialylated bovine colostrum

In vitro phagocytosis assay: mouse peritoneal adherent cells containing macrophages were collected from 8-week-old female ICR mice, as previously reported by Uto et al,[93] and cultured in 24-well plates at a density of 5×105 cells/well in serum-free RPMI 1640 for 1 h. The cultured cells were then washed three times with serum-free RPMI 1640 to separate adherent macrophages from non-adherent cells, such as T and B cells. Mouse peritoneal macrophages were layered onto coverslips in a 24-well plate and cultured for 15 h. Then for the comparative study cells were treated for the 3 h either with 10 ng of degalactosylated bovine colostrum, 10 ng degalactosylated/desialylated bovine colostrum; 10 ng of non-treated bovine, 1,1 μg of LPS (a positive control). The 3 h culturing macrophages with serum-free RPMI 1640 served as negative control. All experiments were performed in triplicate. The cultures were assayed for phagocytic activity. Sheep red blood cells were opsonized by rabbit hemolytic serum (anti-sheep red blood cells, Cosmo Bio Co., Tokyo, Japan). Opsonized SRBCs (0.5%) in serum-free RPMI 1640 were overlaid onto each macrophage-coated coverslip and cultured for 1.5 h. The non-internalized erythrocytes were lysed by immersing the coverslip into a hypotonic solution (1/5-diluted phosphate-buffered saline). The macrophages were fixed with methanol, air-dried and stained with Giemsa stain. The number of phagocytosed erythrocytes per cell was determined microscopically; in total, 250 macrophages were counted for each data point. The data was expressed in terms of the phagocytosis index (PI), which was defined as the percentage of macrophages with ingested erythrocytes multiplied by the mean number of erythrocytes ingested per macrophage.

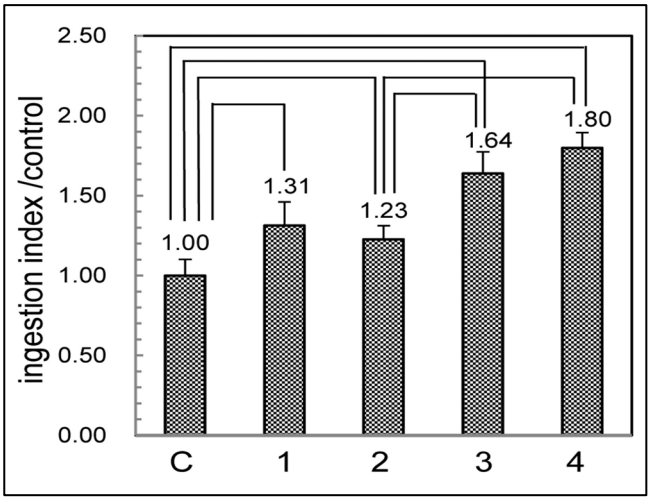


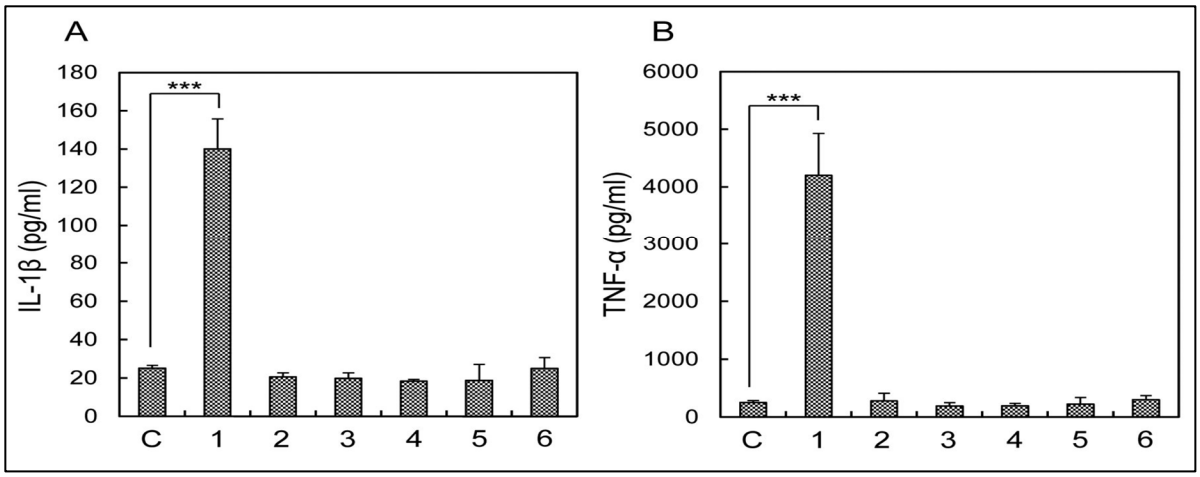
Figure 5
In vitro phagocytic activity of mouse peritoneal macrophages seen under exposure to pure, degalactosylated and degalactosylated/desialylated bovine colostrum
*C, Control; 1, 1 μg of LPS; 2, 10 ng of non-treated bovine colostrum; 3, 10 ng of degalactosylated bovine colostrum; 4, 10 ng of degalactosylated/desialylated bovine colostrum. The number on each bar indicates the mean value. *p<0.05. Each error bar represents the standard deviation.*

Result: Stimulating activity of degalactosylated/desialylated bovine colostrum on phagocytic activity of mouse peritoneal macrophages. We examined phagocytic activation by using degalactosylated and degalactosylated/desialylated bovine colostrum against mouse peritoneal macrophages. Figure 5 shows significant phagocytic activation with 10 ng of degalactosylated and degalactosylated/ desialylated bovine colostrum, compared to that observed with the control, 10 ng of non-treated bovine colostrum showed significant phagocytic activation compared to that observed with the control; however, its activity was relatively weak (PI=1.23).[93]

Degalactosylated/desialylated bovine colostrum and human serum *in vitro* treatment do not induce the inflammatory cytokine synthesis in mouse peritoneal macrophages

Flow cytometry assay. Mouse peritoneal macrophages were cultured in 24-well plates at a density of 5×105 cells/well in serum-free RPMI 1640 for 15 h. The cultured cells were washed two times with serum-free RPMI 1640 and then treated for 24 h either with 1, 1 μg of LPS and 10 ng of interferon-γ (positive control); 10 ng of non-treated bovine colostrum; 10 ng of degalactosylated bovine colostrum; 10 ng of degalactosylated/desialylated bovine colostrum; 10 ng of non-treated human serum; 10 ng of degalactosylated/desialylated human serum; the culturing with serum-free RPMI 1640 was used as negative control. The supernatant (50 μl) was added to a mixture of capture bead diluent (48 μl), mouse IL-1β capture bead E5 (1 μl) and mouse TNF-α capture bead C8 (1 μl) (Becton, Dickinson and Company). After the solution containing beads was incubated at room temperature for 1 h, the mixture of capture bead diluent (48 μl), mouse IL-1β PE detection reagent (1 μl) and mouse TNF-α PE detection reagent (1 μl) were added. The solution containing beads was incubated at room temperature for 1 h, the beads were washed with 1 ml of wash buffer and centrifuged at 200 × g for 5 min. The supernatant was removed and then 300 μl of wash buffer was added and analyzed using FACSVerse.

Figure 6



Mouse peritoneal macrophages inflammatory cytokines (A) IL-1β and (B) TNF-α response on *in vitro* treatment *C, Control; 1, 1 μg of LPS and 10 ng of interferon-γ; 2, 10 ng of non-treated bovine colostrum; 3, 10 ng of degalactosylated colostrum; 4, 10 ng of degalactosylated/ desialylated bovine colostrum; 5, 10 ng of non-treated human serum; 6, 10 ng of degalactosylated/desialylated human serum. All experiments were performed in triplicate. Each error bar represents the standard deviation. The number on each bar indicates mean value. ***p<0.005.*

Result neither IL-1β (A) nor TNF-α (B) the major inflammatory cytokines was secreted by mouse peritoneal macrophages activated by enzymatically treated bovine colostrum and human serum.[93]

2.4.4 In vivo study

Colostrum MAF induces decreasing of IL-6 level in plasma of healthy dogs

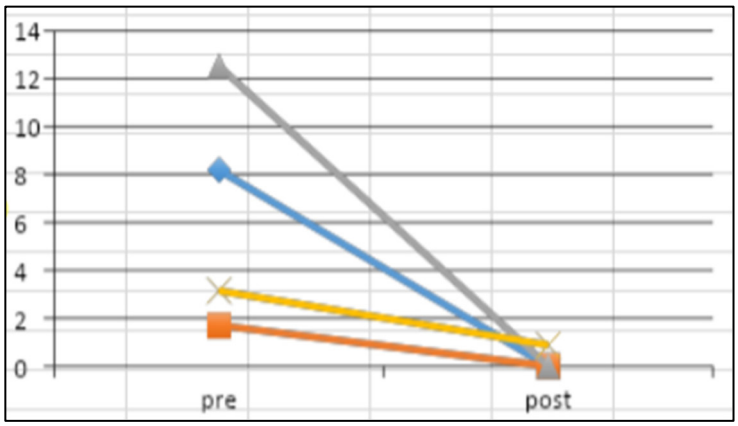


Figure 7

The Saisei colostrum MAF (degalactosylated bovine colostrum) was orally administrated to four healthy dogs in 0.2mg/kg daily dose for 21 days.

Result: The measured by ELISA test IL-6 level was highly variable at baseline 8.19, 1.7, 12.5, and 3.15 pg/mL in the studied group, and it dropped to an undetectable level in 3 animals and to close to the detection threshold in 1 animal on the day 21.

2.4.5 Safety

The primary risks of clinical studies are mainly related to the potential adverse effects associated to the study drug and study procedures.

The active ingredient of Saisei colostrum MAF capsules is the enzymatical treated heat-inactivated bovine colostrum. The used lactase enzyme produced by *Kluyveromyces marxianus* is considered safe by the US FDA. The Saisei Pharma manufacturing facility in Japan is GMP certified for food production. Saisei colostrum MAF contains no live bacterias and no potentially toxic compounds. The organic basis of the studied dietary supplement provides its safety and digestibility comparable to the product's source - fermented bovine colostrum contained in a small - 2.3 mg amount per capsule. There were no adverse reactions reported during 8 years of the product's commercial use. The product's contraindication is the possible allergy to the dairy products; colostrum MAF is not recommended for children under 3 years of age, and there are precautions for use for pregnant and lactating women.

Macrophages activation by other biological macrophage activators known to be often accomplished with a variety of pro-inflammatory effects. In contrast, these cells activation induced by Saisei MAFs is not accompanied by a pro-inflammatory effect *in vitro,* moreover, it resulted in a decrease of a key inflammatory cytokine IL-6 level *in vivo*; and *in vitro* experiments showed that it does not drive the further activation of initially highly activated cells. As a supplement in humans, Saisei colostrum MAF has been mainly used on inflammatory genesis conditions combine treatment. Thus, studied MAF, is rather preventing conditions related to pathological inflammatory macrophage activation and over-activation.

2.4.6 Mechanism of macrophage activation

Purified GcMAF. Purified GcMAF increases macrophage phagocytic activity via Fc-gamma receptor,[41,49] it is also reported to increase Fc receptor-mediated phagocytosis in murine peritoneal macrophages by inducing translocation of FcγRI and FcγRII from the intracellular compartment to the cell surface of macrophages.[95] GcMAF stimulates 3'-5'cyclic adenosine monophosphate formation in human peripheral blood mononuclear cells and stimulates their proliferation.[96] But the detailed mechanism of GcMAF-mediated macrophage activation cascade has not yet been elucidated.

Serum MAF and colostrum MAF produced by Saisei Pharma. Both formulations increase

phagocytosis *in vitro* in primary macrophages as mouse peritoneal and intestinal macrophages and in human monocytic cell lines.[89,91,93,97] Serum MAF has been shown to significantly increase phagocytic index in mouse peritoneal macrophages by up to 43% with a 1 ng dose and by up to 74% with a 10 ng dose. Later when the best quality enzymes and treatment regimes had been determined, 8.17 μg/ml serum MAF was shown to increase the phagocytic index by four times compared to the control (Figure 4). No significant phagocytic activation was seen in peritoneal mouse macrophages incubated with non-treated human serum.[91] The same 10 ng dose of degalactosylated and degalactosylated/desialylated bovine colostrum has been shown to have a similar trend (Figure 5).[93]

On comparative study different macrophage activation factors potency on four types of differentiated macrophages; normally differentiated THP-1 (dTHP1) and U937 (dU937), as well as sensitized THP-1 (sTHP1) and U937 (sU937), the IL-4, IL-10 and LPS+IFN-γ failed to activate sTHP1, dU937 and sU937. However, Saisei serum MAF was able to activate all types except dU937, where phagocytic activity was initially high in the control. Culturing in FBS-free medium turned the fully activated U937 into the basal state and, subsequently, serum MAF was able to activate them too. Although the phagocytic activity of sTHP1 and sU937 were clearly different, IL-4, IL-10 and LPS+IFN-γ were unable to activate either macrophage, whereas serum MAF could significantly activate both.[97] The conclusion was drawn from these results that serum MAF has a different mechanism of activation from the other macrophage activators listed above. It suggests that serum MAF recognizes and activates dTHP1 through the GalNAc moiety of Gc protein, which is one of the numerous components of the activation mechanism.

Almost all of the key molecules involved in the innate and adaptive immune response are glycoproteins. Whole human serum and bovine colostrum are substrates of the Saisei MAFs degalactosylization/desialylation treatment, therefore, apart from GcMAF, there other glycoproteins are abundant. Applied enzymatic treatment could enhance the activity of *O*-linked glycoproteins in the manner seen on Gc protein. It is likely that some of them are synergic to GcMAF and contribute serum and colostrum MAFs higher activity compared to purified GcMAF. Below there are the studies supporting this concept.

Immunoglobulin A (IgA) and other glycoproteins contribute degalactosylated/desialylated human serum and bovine colostrum immunological activities. The macrophage activation property degalactosylated/desialylated biological fluids as bovine colostrum or human plasma/serum are not restricted by GcMAF and at least immunoglobulins with *O*-linked sugar chains are contributing to it. The western blot analysis with the HPA lectin of human serum and

bovine colostrum treated with β-galactosidase and sialidase showed the 70 and 75 kDa bands which may be IgA or IgD heavy chain with *O*-linked oligosaccharides that consist of GalNAc, galactose, and sialic acid with the same sugar chain composition as Gc protein.[91,93] The IgA binding property for Fc receptor is reduced if many sialic acid residues exist. IgA could be activated by sialidase and /or β-galactosidase treatment as the deglycosylated and/or desialylated IgA affinity for FcαRI is shown to be increased.[98] This receptor is involved in numerous processes, including phagocytosis, antibody-dependent cell cytotoxicity and cytokines release. Further investigation is needed to elucidate whether other glycoproteins of human serum and bovine colostrum are also enhanced in activity after this enzymatic treatment.

Synergistic effects of 25-OH vitamin D deglycosylated vitamin D binding protein complex. In biological fluids, the vitamin D binding protein (VDBP) - Gc protein is mainly conjunct with 25(OH) VitD in (VitD~VDBP) complex. The separation of the VitD from VDBP used in the first generation purified GcMAF. Above it was clearly shown that there is a difference in stability and activity between the purified GcMAF and both serum and colostrum MAFs where GcMAF remains VitD bound. This supported by other research where the advanced synergistic effects on recognition, activation, phagocytosis and oxidative stress on macrophages of VitD - Deglycosylated VDBP (VitD~dgVDBP) dimer compared to VitD free Gc protein (VDBP), and also to deglycosylated dgVDBP (GcMAF) have been shown.[99] The recognition of the antibody against VDBP was found significantly more than 4-fold higher for VitD~dgVDBP compared to dgVDBP and 7-fold higher for VDBP. There was a linear regression between VDBP antibody affinity and macrophage phagocytosis resulting in a correlation coefficient of r = 0.95 in favor of VitD~dgVDBP. This could explain the fast membrane remodeling of macrophages seen in 4-5 mins of exposure to serum MAF (Figure 3).[89]

2.5 Procedures Used for prognostic analysis

The MAF precursor activity of serum Gc protein and serum Nagalase activity were determined to show recovery rate of immuno-potency during the early stage of GcMAF therapy. It was found an inverse correlation between the MAF precursor activity and serum levels of nagalase, therefore showing to be other than pathogenicity or disease biomarkers, also good prognosticators of illness and response to therapy.[76-86]

- *MAF precursor activity of serum Gc protein*

Lost or reduced MAF precursor activity of patient serum Gc protein are exhibited as a decrease in superoxide generation macrophages as compared with control healthy human Gc protein (reference value: 0.5–0.8 nmol superoxide/min/10^6 macrophages).[46] Thus, if patient serum (0.1%)

generates < 0.8 nmol superoxide/min/10^6 macrophages, the precursor activity of the patient serum Gc protein is considered to be lost. This procedure measures the ability of individual patient to activate macrophages as for immune potential. Assay procedure for determination of MAF precursor activity of patient serum Gc protein has been described previously. [38,39,46]

- *Nagalase activity*

Healthy control sera exhibit very low levels (reference range: 0.35–0.68 nmol/min/mg) of the enzyme activity of a-galactosidase. Yamamoto N et al[76] have been showed that a reduction in serum Nagalase activity to 0.68 nmol/min/mg or less in patient during GcMAF therapy served as demonstration that HIV-infection has been eradicated.

2.6 Purpose of the study

The purpose of this study is to evaluate the efficacy and safety of immunotherapy with oral MAF plus standard-of-care therapy in hospitalized patients with COVID-19- induced pneumonia. Currently, no approved treatment for COVID-19 is available.

3. Study Objectives and endpoints

Specific objectives and corresponding endpoints for the study are outlined below.

3.1 Primary objective and primary endpoint

The primary efficacy objective for this study is to evaluate the efficacy of oral MAF for the treatment of COVID-19 pneumonia on the basis of the following endpoint:

- The rate of transfer to the intensive care unit (ICU), defined by the proportion of hospitalized patients requiring intensive care management because of worsening respiratory function (PaO_2/FiO_2 ratio <150 mmHg) and/or development of multi-organ dysfunction and/or other clinical conditions needing invasive mechanical ventilation.

Given that 26% of patients required intensive care unit treatment, as previously observed (64), the purpose of this trial is to achieve a reduction of at least 50% of this value with an overall rate of transfer to the ICU of 13%.

3.2 Secondary objective and secondary endpoints

The secondary efficacy objective for this study is to evaluate the efficacy of oral MAF for the treatment of COVID-19 pneumonia on the basis of the following endpoints:

- Changes from baseline to subsequent timepoints (when available) in terms of percentage of lung involvement (lung consolidation, ground glass opacities and disease free). CT visual quantitative evaluation will be based on summing up the acute lung inflammatory lesions involving each lobe, which was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%), respectively. In particular, an early reduction from 85% to 50% in imaging progression on chest CT at day 7 will be evaluated, as a surrogate endpoint;
- disease progression on ultrasound imaging according to Lung Ultrasound (LUS) score;
- duration of hospital stay, expressed in days;
- days on non-invasive ventilation;
- days on mechanical ventilation;
- days in ICU;
- days with use of supplemental O_2;
- discharge rate at day 28;
- clinical evolution;

- time to resolution of fever (for at least 48 hours) in absence of antipyretics, or discharge, whichever is sooner in the 4-week period after study treatment. Resolution of fever is defined as body temperature: ≤36.6°C (axilla) or ≤37.2 °C (oral), or ≤37.8 °C (rectal or tympanic). Fever is defined as defined as body temperature > 37.4°C [axilla], or > 38.0 °C [oral], or >38.4°C [rectal or tympanic];
- progression of respiratory failure as evaluated by SpO_2 and the PaO2/FiO2 ratio in the 28-day period or until discharge, whichever comes first;
- proportion of patients requiring implementation of supplemental oxygen during the 28-day period;
- changes from baseline in: white blood cell count (WBC), hemoglobin, platelets, CRP, ESR, LDH, procalcitonin, IL-1, IL-6, TNF-α, D-dimer, and fibrinogen;
- changes from baseline in: MAF precursor activity of serum Gc protein and serum nagalase activity, as markers for response to treatment;
- kinetic changes of viral loads detected in nasopharyngeal swabs;
- number of Serious Adverse Events (SAE) and Adverse Drug Reaction (ADR) (expected and unexpected) until the discharge from the clinical unit (discharge for any motivation);
- patient compliance with treatment.

4. Study design

This is a phase II, multicenter, prospective, interventional, non-profit study at the COVID Center, Ospedale del Mare, Naples (coordinator center) and at the Department of General Medicine, "San Giovanni Bosco" COVID Hospital, Naples (other investigational center). The trial aims to assess the efficacy and safety of immunotherapy with oral MAF plus standard-of-care therapy in hospitalized adult patients with COVID-19- induced pneumonia. A total of 97 patients who meet the inclusion/exclusion criteria will be enrolled in the study, that will be conducted at the COVID Center, Ospedale del Mare, Naples (coordinator center) and at the Department of General Medicine, "San Giovanni Bosco" COVID Hospital, Naples (other investigational center). Enrollment will stop as soon as the target number of recruited subjects is reached. An informed consent will be obtained from patients before assessments solely required for the study are performed. Thereafter, eligibility criteria will be reviewed by study personnel. Eligible patients will be treated with the stronger version of Saisei MAF capsules 148 mg, oral administration 3 capsules, 3 times per day. The treatment duration will be 21 days. Patients are also provided with nutritional supplementation of Vitamin D3, 10.000 IU per day, monitoring the blood levels of such a vitamin. Efficacy and safety assessments will be performed on Days 0, 1, 3, 5, 7, 14, 21, and 28. Eligible patients will have biomarker sampling according to the Assessment and Procedures Schedule (see below). A follow-up period of 28 days will be considered.
This is a multicenter trial.

4.1 Rationale for study design

This II Phase interventional non-randomized study supports the assessment of efficacy as well as safety of Saisei colostrum MAF plus standard-of-care therapy for patients with COVID-19- induced pneumonia. Currently, no approved treatment for COVID-19 is available. A well-designed phase-II interventional trial allows to evaluate the feasibility and efficacy of new investigational drugs in a short time and provides a basis for planning randomized placebo-controlled trials in order to understand the best and safest treatment options for patients with COVID-19.

4.2 Rationale for Saisei colostrum MAF dose

As mentioned above Saisei Pharma colostrum MAF consists of multiple active ingredients that provide immunomodulating activity. Its therapeutic dose cannot be estimated based solely on the GcMAF content. There is also a lack of a standard method to quantitatively analyze and detect GcMAF. In addition, the issue is that Gc protein and GcMAF have similar Isoelectric focusing mobility in Western blot.[100]

The product's effects are seen at an extremely low dose/concentration. In *in vitro* studies, 10 ng of degalactosylated and degalactosylated/desialylated bovine colostrum increased the phagocytosis index higher than was seen with 1 μg of LPS treatment (Figure 5).[93] It becomes a background for the relatively small amount per capsule of 2.3 mg of total bovine colostrum which consists of around 1 mg of colostrum proteins. The maximum daily dose is 10 capsules, which corresponded to 23 mg bovine colostrum. Saisei Pharma colostrum MAF is formulated as a 148 mg enteric capsule for oral intake. One capsule contains 2.3 mg (1.6%) of active ingredients - the enzymatically treated bovine colostrum.

The recommended daily dose for adults consists of 1-2 capsules for prophylactic purposes and 6 - 10 capsules for immune deficiency and inflammatory conditions. Intake duration is two-three weeks and the course can be repeated within a two-week interval. The dose of 6-10 capsules daily for three weeks in adults have shown the trend to downregulate proinflammatory laboratory markers in conditions associated with chronic inflammation. According to the post-marketing observation this intake regimen has been shown to be well tolerated and has never been associated with side effects or adverse reactions. However, if patients have moderate to severe pneumonia, the recommended dosage can be increased up to 18 capsules of oral colostrum GcMAF / day (6 capsules 3 times a day 30 minutes before food or in the morning, afternoon and before bed time) with almost no side effects, according to Saisei Mirai Pharma. However, a stronger version of oral colostrum GcMAF capsules, which are equivalent to 3 capsules of usual oral colostrum GcMAF, is available for this trial.

4.3 Rationale for Saisei colostrum MAF administration route

Effects on the gastrointestinal tract. Gastrointestinal involvement is well known in coronavirus infections of animals and humans. The angiotensin-converting enzyme II (ACE2), the entry receptor for SARS-CoV, is highly expressed in proximal and distal enterocytes that are directly exposed to foreign pathogens. It considers the mechanism of SARS-CoV-2 can actively infect and replicate in the gastrointestinal tract. SARS-CoV-2 indirectly damages the digestive system through a chain of inflammatory responses. With increasing gastrointestinal wall permeability to foreign pathogens once virus infected, enteric symptoms like diarrhea will occur by the invaded enterocytes resulting in malabsorption.

Multiple colostrum proteins considered effective for the prophylaxis and the therapy of different genesis of intestinal inflammation.[101,102] Reduction of gastrointestinal hyperpermeability by bovine colostrum is described in newborns, children, adults,[101,103] and in animal studies.[104]

Delivered topically to the small intestine by an acid-resistant enteric-coated capsule colostrum MAF can directly activate a large number of gut mucosal macrophages for virus control, localizing intestinal inflammation and resolving through driven phagocytic scavenger function. These and tissue regeneration effects are expected to prompt the restoration of intestinal barrier function.
Macrophages in the gastrointestinal mucosa represent the largest pool of tissue macrophages in the body, which besides the local functions are directing the systemic immune response. They also could modulate the respiratory tract mucosal immunity through immune regulation, the so-called "gut-lung axis".

4.4 Rationale for nutritional supplementation of Vitamin D3

As above described, the main Saisei MAFs active substance - the VitD~dgVDBP complex has a higher macrophage activation and lower oxidative burst than VitD free dgVDBP.[99] VitD can also contribute to different immunological effects of VitD~dgVDBP complex as its receptors are expressed on B and T lymphocytes and antigen-presenting cells.

5. Study population

The study population includes adult male and female patients who are hospitalized and diagnosed with COVID-19-induced pneumonia.

5.1 Inclusion criteria

Participants eligible for inclusion in this protocol must meet **all** of the following criteria:

- Adults (≥ 18 years of age);
- signed informed consent by any patient capable of giving consent, or, when the patient is not capable of giving consent, by his or her legal/authorized representative or according to local guidelines;
- patients clinically diagnosed with SARS-CoV-2 virus by PCR or by other approved diagnostic methodology;
- hospitalized with COVID-19-induced pneumonia evidenced by chest x-ray or CT scan with pulmonary infiltrates;
- patients having a PAO2/FIO2 ratio > 250 mmHg
- well-selected patients having a PAO2/FIO2 ratio ≤ 250 mmHg that, in the investigator's judgment, doesn't preclude the patient's safe participation in and completion of the study;
- patients being able to swallow

5.2 Exclusion criteria

- Proportion of hospitalized patients requiring invasive mechanical ventilation at the time of hospital admission (patients requiring non-invasive mechanical ventilation are eligible);
- onset of COVID-19 pneumonia symptoms (i.e. dyspnea/respiratory insufficiency) > 14 days
- uncontrolled systemic infection (other than COVID-19);
- any prior (within the defined periods below) or concurrent use of immunosuppressive therapies including anti-IL-6, anti-IL-6R antagonists or Janus kinase inhibitors (JAKi) in the past 30 days or plans to receive during the study period;
- hypersensitivity to the active substance or to any of the excipients of the experimental drug, including known allergy to dairy product;

- any serious medical condition or abnormality of clinical laboratory tests that, in the investigator's judgment, precludes the patient's safe participation in and completion of the study;
- in the opinion of the investigator, progression to death is imminent and highly likely within the next 24 hours, irrespective of the provision of treatments;
- current participation in any other interventional investigational trials;
- pregnant or breastfeeding woman;
- concurrent malignancy requiring chemotherapy;
- renal insufficiency;
- all types of disability.

6. Treatment plan

The study will enroll patients who require hospitalization with supportive care for COVID19 pneumonia, including supplemental oxygen. All patients will receive, in addition to the best available standard of care, colostrum MAF via oral.

Given the known immunomodulatory properties of Vitamin D3, and bearing in mind that GcMAF is a component of the Vitamin D axis, patients are also provided with nutritional supplementation of Vitamin D3, 10.000 IU per day, monitoring the blood levels of such a vitamin.

6.1 Investigational drug

Dietary supplement name: Colostrum MAF, Saisei MAF

Reported activity: immunomodulator

Formulation: 148 mg acid-resistant coated capsules, containing 2.3 mg of enzymatically treated bovine colostrum powder and supplementary ingredients

The dietary supplement substances:

Active ingredient	Enzymatically treated bovine colostrum powder	2.3 mg	1.6 %
Supplementary ingredients	Lactase (Derived from yeast)	0,15 mg	0.1 %
	HPMC (Hydroxypropyl Methylcellulose) acid-resistant capsule	47 mg	31.8 %
	Microcrystalline cellulose (Derived from pulp)	98,4 mg	66.5 %

Dosage for adults: 2 - 10 capsules daily

Dosage for children after 3 years old: 2-6 capsules daily

Route of administration: oral

Contraindication: allergy to dairy product components

Precaution: pregnancy and lactation

Storage: Can be stored in at + 5 to +25°C, on dry place for up to two years

Manufacturer: Saisei Pharma, Osaka, MORIGUCHI city, OKUBO-cho, 3-34-8. Japan

All patients will be treated with the stronger version of **Saisei MAF capsules 148 mg**, oral administration 2-3 capsules, 3 times per day, 30 minutes before food or in the morning, afternoon and before bed time. If necessary (moderate to severe COVID-19), the dosage will be increased according to individual requirements up to a maximum of 18 capsules daily (6 capsules, 3 times a day) or 3 capsules (stronger version), 3 times a day.
The treatment duration will be 21 days.
Patients are also provided with nutritional supplementation of **Vitamin D3, 10.000 IU per day**, monitoring the blood levels of such a vitamin.

6.2 Concomitant treatments

All medications, procedures, and significant non-drug therapies (including but not limited to pronation, physical therapy, and blood transfusions) administered after the participant was enrolled into the study must be recorded on the appropriate CRF. Each concomitant drug must be individually assessed against all exclusion criteria/prohibited medication. If in doubt, the investigator should contact the Promoter before enrolling a participant or allowing a new medication to be started. If the participant is already enrolled, contact the Promoter to determine if the participant should continue participation in the study. The patient must be told to notify the Treating Physician about any new medications that he/she takes after the start of Colostrum-MAF, also after potential discharge from the hospital.

7. Informed consent procedures

Eligible participants may only be included in the study after providing Independent Ethics Committee IEC-approved informed consent. If applicable, in cases where the participant's representative(s) gives consent (if allowed according to local requirements), the participant must be informed about the study to the extent possible given his/her understanding. If the participant is capable of doing so, he/she must indicate agreement by personally signing and dating the written informed consent document. Informed consent must be obtained before conducting any study-specific procedures (e.g. all of the procedures described in the protocol). The acquisition of informed consents and information forms should be documented in the patient's medical records, as required by ICH GCP, and the information and informed consent forms should be signed and personally dated by the patient, or a legally acceptable representative, and by the physician who conducted the information and informed consent discussion. The original signed information and informed consent forms should be retained in accordance with institutional policy, and a copy of the signed forms should be provided to the patient or legally acceptable representative. All patients will be informed of the aims of the study, the potential benefit, the possible adverse events, the procedures and possible hazards to which he/she will be exposed, and the mechanism of treatment. It will be emphasized that the participation is voluntary and that the patient is allowed to refuse further participation in the protocol, whenever he/she wants. This will not prejudice the patient's subsequent care. They will be informed as to the strict confidentiality of their data, but that their medical records may be reviewed for trial purposes by authorized individuals other than their treating physician. This must be done in accordance with the local regulatory requirement Regulatory authorities and/or IEC may request access to all source documents, data capture records, and other study documentation for one-site audit or inspection. Direct access to these documents must be guaranteed by the investigator, who must provide support at all times for these activities. The patient's confidentiality will be maintained and will not be made publicly available to the extent permitted by the applicable laws and regulations (Low n. 675/1996 and amendments) and Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46/EC (General Data Protection Regulation). The name of the patient will not be asked by the promoter. An identification number will be automatically attributed to each patient enrolled in the trial. This number will identify the patient and must be included on all case report forms. In order to avoid identification errors, date of birth will also be reported on forms. Information about common side effects already known about the investigational drug can be found in the Investigator's Brochure (IB). This information is included

in the participant informed consent and should be discussed with the participant during the study as needed. Any new information regarding the safety profile of the investigational drug that is identified between IB updates will be communicated. New information might require an update to the informed consent and then must be discussed with the participant. Women of childbearing potential must be informed that taking the study treatment may involve unknown risks to the fetus if pregnancy were to occur during the study and agree that in order to participate in the study they must adhere to the contraception requirements.

8. Visit schedule and assessments

The Assessment & Procedures Schedule (Table 1) lists all of the assessments when they are performed. All data obtained from these assessments must be supported in the participant's source documentation. Participants should be seen or phoned for all visits/assessments as outlined in the assessment schedule (Table 1) or as close to the designated day/time as possible. Missed or rescheduled visits should not lead to automatic discontinuation. At the day of discharge from the hospital, all assessments in Table 1 should be conducted. In addition, contact information for the patient and/or legal representatives should be obtained for the follow-up visits. Screening assessments are to be taken as indicated in Table 1 prior to dosing and are considered baseline measurements. Patients who meet the inclusion/exclusion criteria may be treated immediately after obtaining the baseline measurements

8.1 Schedule of Assessment and Procedures (table 1)

Visit Day	**Screening**	**0 (start of study drug administration)**	**1**	**Every day during hospitalization until day 28 or discharge (whichever is earlier)**	**3°**	**5°**	**7°**	**14°**	**21°**	**28 (or at discharge)**
Informed consent	x									
Inclusion/esclusion criteria	x	x (p)								
Demographics /medical history / relevant comorbidities	x									
SARS-CoV-2	x	RECORD ALL SARS-CoV-2 DETERMINATIONS AS OBTAINED								
Vital signs (HR, RR, SpO_2, $PaCO_2$, PaO_2, BP, body temp.)	x	x (p)	x	x	x	x	x	x	x	x
Oxygen delivery (flow + FiO2)	x	x(p)	x	x	x	x	x	x	x	x
NIV use (hours/day)	x	x(p)	x	x	x	x	x	x	x	x
Vital status (and if applicable, cause of death)			x	x	x	x	x	x	x	x
Blood cultures (Aer/Anaer)		X								
Hematology (Hb, MCV, WBC, Plt, absolute count of N,L,M,E,B)	x	x(p)	x	x	x	x	x	x	x	x
Clinical chemistry (AST, ALT, ALP, LDH, creatine kinase, troponin, creatinine, bilirubin, glucose, total protein, CRP, ferritin, D-dimer, fibrinogen, PCT; IL-1, IL-6, TNF-α [if available])	x	x(p)	x	x	x	x	x	x	x	x
Pregnancy test (if applicable)	x									
CT scan /chest X-ray	x		RECORD ALL PULMONARY IMAGING AS OBTAINED							X
Adverse events	x	x	x	x	x	x	x	x	x	x
Prior/concomitant medications	x	x(p)	x	x	x	x	x	x	x	x
Study drug administration		x					x	x		
In-hospital outcomes • *Days in hospital* • *Days with use of supplemental O_2* • *Days on non-invasive ventilation* • *Days on mechanical ventilation* • *Days in ICU* • *Discharge*		x(p)	x	x	x	x	x	x	x	x
Blood collection for experimental outcomes		x(p)					X	X	X	

X If possible

(p) All assessment at day 0 must be conducted prior to colostrum-MAF administration

8.2 Participant demographics/other baseline characteristics

Age, sex, race, ethnicity, anthropometric data, medical history, cardiovascular risk factors, relevant comorbidities, pre-existing therapy.

8.3 Efficacy

Vital signs and oxygen saturation

Vital sign measurements include respiratory rate, pulse rate, systolic and diastolic blood pressure, and body temperature. Peripheral oxygen saturation should also be measured at the same time as the vitals. For patients requiring supplemental oxygen, the oxygen flow rate (L/min) and/or fraction of inspired oxygen (FiO2) should be recorded.

Laboratory evaluations

Laboratory evaluations will be performed by the local lab.

Arterial Blood Gas (ABG) analysis

SpO_2, $PaCO_2$, PaO_2, pH, P/F, and lactate will be measured according to the assessment schedule in Table 1.

Hematology

Hemoglobin, mean corpuscular volume (MCV), white blood cell count with differential, and platelet count will be measured according to the assessment schedule in Table 1.

Chemistry

Creatinine, total bilirubin, direct bilirubin, AST, ALT, alkaline phosphatase, lactate dehydrogenase, creatine kinase, troponin, total protein, glucose will be measured according to the assessment schedule in Table 1.

If a given test will be not available locally, this will be documented on the eCRF.

Additional markers of inflammation: C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), lactate dehydrogenase (LDH), procalcitonin (PCT), ferritin, D-dimer, IL-6, fibrinogen, IL-1, IL-6, and TNF-α (if locally available) should be collected in accordance with Table 1.

In the case where a laboratory range is not specified by the protocol, but a value is outside the reference range for the laboratory at screening and/or initial baseline, a decision regarding whether

the result is of clinical significance or not shall be made by the Investigator (in consultation with the Promoter) and shall be based, in part, upon the nature and degree of the observed abnormality. In all cases, the Investigator must document in the source documents, the clinical considerations (i.e., result was/was not clinically significant and/or medically relevant) in allowing or disallowing the participant to continue in the study. All patients with laboratory tests containing clinically significant abnormalities should be followed until the values return to within the normal ranges or until a clinical explanation is identified, even after study medication has discontinued.
Laboratory evaluations will be performed by the local lab.

Chest x-ray or CT scan

Standard chest x-ray (PA view) or CT scan will be performed for eligibility except for those who have had a valid x-ray or CT scan done within 4 days prior to first dosing. The results must be known prior to recruitment to determine the subject's eligibility for the study. Additional chest x-rays (or CT scan) will be performed, as needed. Radiological findings will be assessed according to the aforementioned visual score, in relation to the evidence of ground-glass opacities, consolidation, peripheral and/or peribronchovascular distribution, interlobular septal thickening within the lesions, air bronchograms, fibrotic lesions, and pleural effusion. Chest x-ray or CT scan results will be recorded in the CRF. However, based on the documented correlation between CT score and LUS score, the excessive radiation exposure and the mandatory scanner disinfection procedures that have to take place, and overall, because of the huge number of critical patients who arrived at the hospital, we reserve the possibility of evaluating lung imaging progression by using the LUS score protocol in addiction to or instead of chest CT evaluation.

In-hospital outcomes

In addition to the endpoints mentioned above, the following in-hospital outcomes will be captured on eCRF(s):

- Days in hospital
- Days on non-invasive ventilation
- Days on mechanical ventilation
- Days in ICU
- Days with use of supplemental O_2
- Time to resolution of fever
- Discharge
- Death.

Patient compliance with treatment

- Dosage, effectiveness, and duration of treatment.

Rate of adherence will be reported as the percentage of the prescribed doses of the medication taken by the patient over the specified period. It will be assessed by using a 3-category ordinal scale and recorded as *high* as ≥ 70%, as *low* as 30-70%, and as *very low* as < 30%.

Presence of SARS-CoV-2 virus

For the Screening inclusion criterion, SARS-CoV-2 virus should be measured by RT-PCR or by other approved diagnostic methodology ≤ 7days of Screening by local lab. Nasopharyngeal swabs will be obtained at baseline and on days 1, 3, 5, 7, 14, 21, 28 (if available) for evaluating kinetic changes of viral loads by using the Threshold Cycle (CT value).

8.4 Additional assessments

MAF precursor activity of serum Gc protein and serum Nagalase activity

In order to detect MAF precursor activity of serum Gc protein and serum nagalase activity, blood samples will be taken at baseline and after 7, 14, and 21 days of treatment (if available) and stored at -20°C.

9. Study discontinuation and completion

Discontinuation of study treatment for a participant occurs when study treatment is stopped earlier than the protocol planned duration and can be initiated by either the participant or the investigator. The investigator must discontinue study treatment for a given participant if, he/she believes that continuation would negatively impact the participant's well-being. Study treatment must be discontinued under the following circumstances:

• Participant/guardian decision

• Use of prohibited treatment as per recommendations in the prohibited treatment section

• Any situation in which study participation might result in a safety risk to the participant

• Following emergency unblinding

• Emergence of adverse events that in the judgment of the investigator, taking into account the participant’s overall status, prevent the participant from continuing participation in the study

• Any laboratory abnormalities that in the judgment of the investigator, taking into consideration the participant’s overall status, prevents the participant from continuing participation in the study

• Severe hypersensitivity reaction occurs, including any of the following: anaphylaxis, fever, chills, urticaria, dyspnea, headache, myalgia, hypotension. Immediate interruption of the infusion to administer study treatment is required in such cases. If discontinuation of study treatment occurs, the investigator should make a reasonable effort to understand the primary reason for the participant’s premature discontinuation of study treatment and record this information. Participants who discontinue study treatment or who decide they do not wish to participate in the study further should NOT be considered withdrawn from the study UNLESS they withdraw their consent. Where possible, they should return for the assessments indicated in the Assessment Schedule. If they fail to return for these assessments for unknown reasons, every effort (e.g. telephone, e-mail, letter) should be made to contact the participant/predesignated contact as specified in the lost to follow-up section. This contact should preferably be done according to the study visit schedule. If the participant cannot or is unwilling to attend any visit(s), the site staff should maintain regular telephone contact with the participant, or with a person pre-designated by the participant. This telephone contact should preferably be done according to the study visit schedule. After study treatment discontinuation, at a minimum, in abbreviated visits, the following data should be collected at clinic visits or via telephone/email contact:

• New / concomitant treatments

• Adverse Events / Serious Adverse Events

The investigator must also contact the IRT to register the participant's discontinuation from study treatment. If discontinuation occurs because treatment code has been broken, please refer to Emergency breaking of treatment code section.

10. Safety monitoring and reporting

10.1 Definition of adverse events and reporting requirements

According to Regulation (EU) No 536/2014 of the European Parliament and of the council of 16 April 2014 for definition of adverse events, adverse reactions and reporting including causality. For the purpose of this protocol adverse events are classified into the following categories:

Adverse events

An adverse event (AE) is any untoward medical occurrence (e.g. any unfavorable and unintended sign [including abnormal laboratory findings], symptom or disease) in a clinical investigation participant after providing written informed consent for participation in the study. Therefore, an AE may or may not be temporally or causally associated with the use of a medicinal (investigational) product. The investigator has the responsibility for managing the safety of individual participant and identifying adverse events. The Promoter will be readily available to advise on trial related medical questions or problems. The occurrence of adverse events must be sought by non-directive questioning of the participant at each visit during the study. Adverse events also may be detected when they are volunteered by the participant during or between visits or through physical examination findings, laboratory test findings, or other assessments. Adverse events must be recorded under the signs, symptoms, or diagnosis associated with them, accompanied by the following information (as far as possible):

1. Severity grade:

- mild: usually transient in nature and generally not interfering with normal activities
- moderate: sufficiently discomforting to interfere with normal activities
- severe: prevents normal activities

2. Its relationship to the study treatment. If the event is due to lack of efficacy or progression of underlying illness (i.e. progression of the study indication) the assessment of causality will usually be 'Not suspected.' The rationale for this guidance is that the symptoms of a lack of efficacy or progression of underlying illness are not caused by the trial drug, they happen in spite of its administration and/or both lack of efficacy and progression of underlying disease can only be evaluated meaningfully by an analysis of cohorts, not on a single participant

3. Its duration (start and end dates) or if the event is ongoing, an outcome of not recovered/not resolved must be reported

4. Whether it constitutes a serious adverse event (SAE) and which seriousness criteria have been met

5. Action taken regarding with study treatment. All adverse events must be treated appropriately. Treatment may include one or more of the following:

- Dose not changed
- Dose Reduced/increased
- Drug interrupted/withdrawn

6. Its outcome (i.e. recovery status or whether it was fatal) Conditions that were already present at the time of informed consent should be recorded in medical history of the participant. Adverse events (including lab abnormalities that constitute AEs) should be described using a diagnosis whenever possible, rather than individual underlying signs and symptoms. Adverse event monitoring should be continued to the end of study visit. Once an adverse event is detected, it must be followed until its resolution or until it is judged to be permanent (e.g. continuing at the end of the study), and assessment must be made at each visit (or more frequently, if necessary) of any changes in severity, the suspected relationship to the interventions required to treat it, and the outcome. Information about adverse drug reactions for the investigational drug can be found in the Investigator's Brochure (IB). Abnormal laboratory values or test results constitute adverse events only if they fulfill at least one of the following criteria:

- they induce clinical signs or symptoms
- they are considered clinically significant
- they require therapy

Clinically significant abnormal laboratory values or test results must be identified through a review of values outside of normal ranges/clinically notable ranges, significant changes from baseline or the previous visit, or values which are considered to be non-typical in participant with the underlying disease.

Serious adverse events

An SAE is defined as any adverse event [appearance of (or worsening of any pre-existing)] undesirable sign(s), symptom(s), or medical conditions(s) which meets any one of the following criteria:

- fatal
- life-threatening

Life-threatening in the context of a SAE refers to a reaction in which the participant was at risk of death at the time of the reaction; it does not refer to a reaction that hypothetically might have caused death if it were more severe (please refer to the ICH-E2D Guidelines).

- results in persistent or significant disability/incapacity

- constitutes a congenital anomaly/birth defect
- requires inpatient hospitalization or prolongation of existing hospitalization, unless hospitalization is for: • routine treatment or monitoring of the studied indication, not associated with any deterioration in condition
- elective or pre-planned treatment for a pre-existing condition that is unrelated to the indication under study and has not worsened since signing the informed consent
- social reasons and respite care in the absence of any deterioration in the participant's general condition
- treatment on an emergency outpatient basis for an event not fulfilling any of the definitions of a SAE given above and not resulting in hospital admission is medically significant, e.g. defined as an event that jeopardizes the participant or may require medical or surgical intervention to prevent one of the outcomes listed above.

Medical and scientific judgment should be exercised in deciding whether other situations should be considered serious reactions, such as important medical events that might not be immediately life threatening or result in death or hospitalization but might jeopardize the participant or might require intervention to prevent one of the other outcomes listed above. Such events should be considered as "medically significant." Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm, blood dyscrasias, or convulsions that do not result in hospitalization or development of dependency or abuse (please refer to the ICH-E2D Guidelines). All new malignant neoplasms will be assessed as serious under "medically significant" if other seriousness criteria are not met. Any suspected transmission via a medicinal product of an infectious agent is also considered a serious adverse reaction. All reports of intentional misuse and abuse of the product are also considered serious adverse event irrespective if a clinical event has occurred.

SAE reporting

To ensure participant safety, every SAE, regardless of causality, occurring after the participant has provided informed consent and until the last study visit must be reported to the Promoter and to the DSMB safety within 24 hours of learning of its occurrence. All follow-up information for the SAE including information on complications, progression of the initial SAE and recurrent episodes must be reported as follow-up to the original episode within 24 hours of the investigator receiving the follow-up information. An SAE occurring at a different time interval or otherwise considered completely unrelated to a previously reported one must be reported separately as a new event. If the SAE is not previously documented in the Investigator's Brochure or Package Insert (new

occurrence) and is thought to be related to the study treatment, an associate from the Chief Medical Office and Patient Safety (CMO & PS) Departments from Kiniksa Pharmaceuticals may urgently require further information from the investigator for health authority reporting. Kiniksa Pharmaceuticals may need to issue an IN to inform all investigators involved in any study with the same study treatment that this SAE has been reported. Reported. The Investigator or the Promoter will voluntary report such events (serious, related) to Kiniksa within 24 hours of awareness. Suspected Unexpected Serious Adverse Reactions (SUSARs) will be collected and reported to the competent authorities and relevant ethics committees in accordance with EU Guidance 2011/C 172/01 or as per national regulatory requirements in participating countries. Any SAEs experienced after the last study visit should only be reported to Novartis Safety if the investigator suspects a causal relationship to study treatment.

10.2 Safety Evaluation

The end of enrollment will be based on interim results, with continuation of enrollment or discontinuation of the study according to the response of patients to treatment. The follow-up according to the protocol is 28 days for each patient enrolled. The end of the study, including statistical analysis and drafting of the final report, is expected at 1 month from the last follow-up of the last patient enrolled. The study will be performed in approximately 3 months starting from the first patient enrolled (depending on the speed of enrollment).

11. Statistical considerations and data analysis

11.1 Determination of sample size

The sample size estimation for this study is based on the primary efficacy endpoint, the rate of transfer to the ICU. A single-stage design as described by A'Hern was used to calculate the sample size. A sample size of 97 patients was considered sufficient to give an 80% probability of rejecting a rate of transfer to the ICU of 26% or more with an exact 1% one-tailed significance test when the true response rate is less than 13%.

The hypothesis being tested will be denoted as follows:

H0: * ≥0.26 vs. H1: * ≤13, where * indicates the proportion of patients admitted to the ICU in the target population.

11.2 Statistical analysis

The analysis of all primary and secondary endpoints will be based on the Evaluable Population (EP) which includes all enrolled subjects who will receive at least one dose of study drug.

Statistical analyses will be performed in R version 3.5.1 (R Project for Statistical Computing) or later. Descriptive statistics will be used to describe the basic features of the data in the study. Patients' clinical characteristics will be summarized with the mean, median, mode, and the minimum and maximum values if continuous and as counts and percent if categorical.

The rate of transfer to the ICU will be calculated by dividing the number of total patients admitted to the ICU by the number of all EP. A 99% Confidence Interval will be calculated to verify the hypothesis that the true rate is less than 26%.

Kaplan–Meier survival curves will also be provided to analyze "time-to-event" data, including mortality, discharge, and imaging progression on chest CT. Kaplan–Meier survival analysis will start by setting the baseline observation of each participant as time 0 (start of the investigational drug administration). The cumulative survival incidence rate will be also determined.

Longitudinal data analysis with mixed models will be used to evaluate laboratory findings. Incidence rate of treatment emergent adverse events will be reported per patient.

Descriptive statistics will be done for safety assessments.

12. Ethical and legal aspects

The study will be conducted according to the principles of Good Clinical Practice (GCP) as reported in current Italian and European legislation. The responsible investigator will ensure that this study is conducted in agreement with the declaration of Helsinki and the Italians laws and regulations, whichever provides the greatest protection of the patient. The protocol has been written and the study will be conducted according to the ICH Harmonized Tripartite Guideline for GCP, issued by the European Union. The relevant Ethical Committee approval must be obtained before starting the trial. A copy of the patient informed consent form must be submitted to the appropriate authority or committee, together with the protocol for written approval. Written approval of the protocol and informed consent by the responsible and appropriate authority or committee must be obtained prior to recruitment of patients to the study. The investigator must inform the appropriate authority or committee of subsequent protocol amendments, which must be approved by this one.

13. Publication of study results

Study Author together with Principal Investigator and the other Investigators will publish the final results of the trial as abstracts to national and international conferences and full manuscript.

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